RHIZOBACTERIAL DETERMINANTS MEDIATED ELICITATION OF PLANT DEFENSE MECHANISMS AGAINST FUNGAL PHYTOPATHOGENS

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ANUSHREE KAMATH

(Reg. No.: IAR/11603)

UNDER THE GUIDANCE OF

DR. DHARA PATEL

ASSISTANT PROFESSOR

DEPARTMENT OF BIOTECHNOLOGY AND BIOENGINEERING

INSTITUTE OF ADVANCED RESEARCH,

GANDHINAGAR, GUJARAT, INDIA

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General Overview

Plant diseases have long posed significant challenges in agriculture, with both abiotic and biotic factors impacting global crop productivity. Phytopathogens, which invade plants and utilize their stored resources, are a major cause of these diseases. Plants possess an innate ability to resist pathogen infection, activating defense mechanisms and providing protection against future infections. This resistance includes the secretion of antimicrobial compounds and other stress responses, which enable plants to recognize and defend against pathogens effectively.

One of the natural defense mechanisms in plants involves the action of PGPR. These beneficial bacteria colonize the rhizosphere and regulate plant growth by enhancing nutrient availability, producing growth hormones, suppressing phytopathogens, releasing volatile compounds, secreting antimicrobial metabolites, and promoting plant defense responses. PGPR can trigger Induced Systemic Resistance (ISR) and/or Systemic Acquired Resistance (SAR), both of which enhance the ability of plant to resist pathogens. There are various biocontrol methods that involve antagonist activity against pathogens or using bioinoculants to protect plants. However, an increase in soil fertility can sometimes reduce the effectiveness of bioinoculants by limiting their ecological niches.

PGPR control pathogen damage through several mechanisms, including siderophore secretion, physical displacement, and the production of antibiotics, enzymes, and other molecules that inhibit the growth of phytopathogens. These beneficial bacteria secrete extracellular enzymes like cellulases, chitinases, lipases, β-1-3-glucanases, and proteases, which degrade polymeric compounds such as proteins, chitin, and cellulose, thus hindering pathogen growth. These enzymes, along with antibiotics, play a critical role in defending against pathogenic fungi, including *Sclerotium rolfsii*, *Botrytis cinerea*, *Phytophthora spp.*, *Cercospora spp., Fusarium oxysporum*, *Pythium ultimum*, and *Rhizoctonia solani*.

In addition to enzymatic action, PGPR also induce ISR by fortifying the physical and mechanical barrier of plant cell walls and modulating the

physiological and biochemical responses. These changes lead to the production of defense chemicals that help the plant resist pathogen attacks. Several studies over the past three decades have demonstrated the positive effects of PGPR on plant development and disease resistance. The induction of systemic resistance involves a specific recognition mechanism between the plant and the PGPR. This interaction enhances the activities of stress-related enzymes such as peroxidase, glucanases, polyphenol oxidase, chitinase, and phenylalanine ammonia-lyase (PAL), as well as increases the content of total phenolic compounds in PGPR-treated plants. PAL is essential for phenolic biosynthesis, and oxidative enzymes like polyphenol oxidase and peroxidase play vital roles in lignifying plant tissues in response to stress.

Key bacterial determinants in ISR include compounds like siderophores, which play a critical role in PGPR-mediated defense. Siderophores are small molecules that bind and sequester iron, depriving pathogens of this essential nutrient and reducing their growth. By producing siderophores, PGPR not only limit pathogen growth but also enhance plant resistance by triggering biochemical responses, including the activation of defense enzymes. The ability of PGPR to produce siderophores is central to their role in promoting plant health and inducing systemic resistance.

Objectives of the study

- Isolation and characterization of rhizobacteria for their biocontrol attributes and root colonization ability property
- Characterization of rhizobacteria for their plant growth promoting traits
- *In planta* studies to check the efficacy of root colonization ability of screened isolates
- Deciphering siderophore dynamics: extraction, purification, and their impact on defense enzyme activity and gene expression modulation
- AI-driven insights: forecasting disease severity of Cercospora leaf spot disease in *Vigna radiata*

Present Investigation

Two rhizobacterial isolates were screened on the basis of their *In vitro* biocontrol potential against mung bean plant pathogen *Cercopsora canescens* plant growth promoting (PGP) activity and root colonizing ability from the soil sample collected from Vagadkhol, Ankleshwar, Gujarat. Among the two isolates, A1 and D5, the isolate D5 being superior was further identified through 16S rRNA sequencing. The sequence was subsequently deposited in GenBank, and an accession number was assigned OQ536061. Influence of bioinoculant isolate A1 and *Bacillus amyloliquefaciens* D5 on the vegetative growth of mung bean plants and the biocontrol efficacy against Cercospora Leaf Spot (CLS) showed that isolate D5 was efficient in enhancing the vegetative growth and disease reduction in plants. The isolate D5 was selected for further development as a bioformulation due to its ability to enhance plant growth parameters and reduce disease incidence. Bioformulations of D5 were prepared using talc, charcoal, cocopeat, and chitosan as carriers, and the shelf life of each formulation was evaluated over a period of six months. To evaluate the elicitor potential of the siderophore, the siderophore was extracted from isolate D5 and purified using Amberlite XAD-4 resin and further characterized to elucidate the structural and functional properties. The purified siderophore was applied to assess its effects on plant growth promotion, disease incidence reduction, and defense enzyme activity. Additionally, native and SDS-PAGE analysis was conducted to examine the induction of defense responses upon treatment with the only siderophore and in combination with *C. canescens* which showed enhancement in defense reactions in presence of pathogen as well as only siderophore treatments. Relative gene expression study was also carried out using purified siderophores which showed enhancement of defense gene expression highlighting the elicitor properties of siderophore against *C. canescens.* A comparison between two AI model random forest and multilayer perceptron was also carried out predicting their accuracy in evaluating the disease severity. This highlights the significant potential of

AI in revolutionizing agricultural practices by enabling precise, efficient, and sustainable disease management.

Chapter 1- Introduction

Plant diseases, caused by abiotic and biotic factors, significantly impact global crop productivity. Phytopathogens exploit plant resources, but plants counter these invasions with innate defense mechanisms, such as antimicrobial secretion and stress responses. These defenses are enhanced by PGPR, which colonize the rhizosphere, improve nutrient availability, produce growth hormones, and suppress pathogens. Among fungal diseases, Cercospora leaf spot disease caused by *Cercospora canescens* is a common foliar disease in mung bean, affecting crops in various regions worldwide. This pathogen can significantly reduce crop yield, causing damage to leaves and leading to defoliation, which may result in substantial yield losses if not properly managed. To address this issue, the application of rhizobacteria as biocontrol agents has emerged as a promising approach to improve crop production. Rhizobacteria are known to induce systemic resistance in plants by enhancing the activities of defense-related genes that code for a variety of proteins. These defense-related proteins include enzymes that regulate secondary metabolism, pathogenesis-related proteins (PR), and regulatory proteins that control the expression of other defense-related genes. The PR proteins include polyphenol oxidase (PPO) and peroxidase (POD), which catalyze the formation of lignin, and phenylalanine ammonia-lyase (PAL), which is involved in the synthesis of phytoalexins and phenolic compounds. Additionally, PR proteins like β-1,3-glucanases (PR-2 family) and chitinases (PR-3 family) degrade the fungal cell wall, leading to fungal cell lysis.

The role of bacterial determinants in rhizobacteria-induced systemic resistance against plant diseases has been studied for over two decades. One key bacterial trait contributing to plant disease suppression is siderophore production. Siderophores are molecules produced by

rhizobacteria to sequester iron from the soil, limiting the availability of this essential nutrient for pathogens like *Cercospora canescens*. By depriving the pathogen of iron, siderophores not only reduce its growth but also trigger enhanced defense responses in the plant, including the activation of early defensive enzymes and the expression of defense-related genes. This highlights the critical role of siderophores in augmenting plant resistance against *Cercospora canescens* and other phytopathogens. Additionally, the application of artificial intelligence (AI) models has shown great promise in improving the management of Cercospora leaf spot disease. AI tools, such as machine learning algorithms, can analyze plant health data to predict disease outbreaks, monitor disease progression, and optimize the timing of biocontrol applications. By integrating AI with biocontrol strategies, such as siderophore application, it is possible to enhance disease management, reduce the reliance on chemical treatments, and improve overall crop yield.

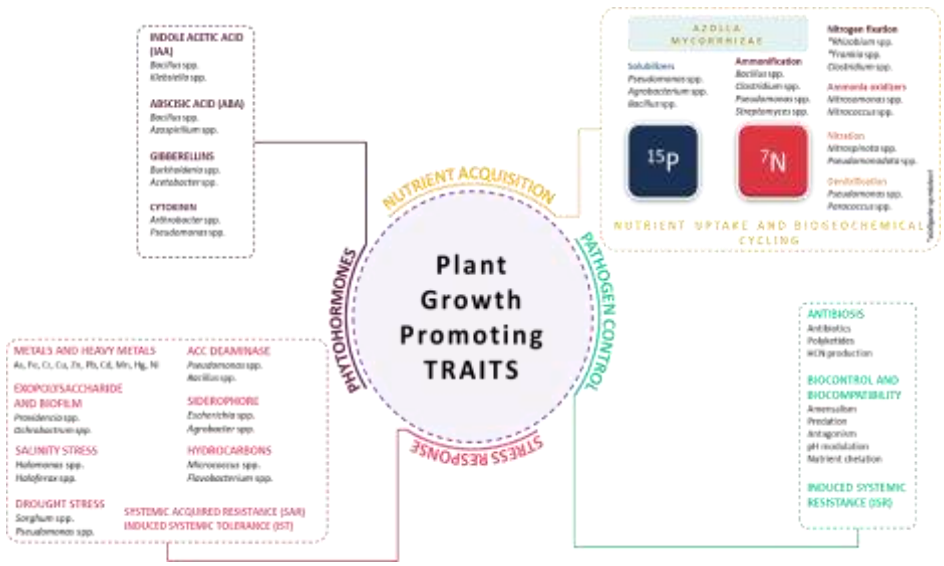


Figure 1.1: Schematic representation of various traits of PGPRs (Kamath et al., 2023)

Chapter 2- Isolation and Characterization of rhizobacteria for their biocontrol attributes and root colonization property

Rhizobacteria were isolated from the rhizosphere of mung bean plants in the Vagadkhol area near Ankleshwar, Gujarat. The physiochemical characteristics of the soil was also studied along with the colony and morphological characteristics of the isolates. From a total of 28 isolates, 14 motile isolates were selected for further analysis. These isolates were screened for their ability to produce siderophores and exopolysaccharides (EPS). Among them, 5 isolates showed individual EPS and siderophore production, and 2 isolates exhibiting both were evaluated for their biocontrol potential against *Cercospora canescens*. The selected isolates A1 and D5 were checked for hydrolytic enzyme production (chitinase, cellulase) and hydrogen cyanide (HCN) production. In dual culture assays, isolates A1 and D5 achieved 71% and 69% pathogen inhibition, respectively. Both isolates demonstrated biofilm-forming abilities, with scanning electron microscopy (SEM) revealing superior root colonization by isolate D5. These findings highlight the biocontrol potential of the selected rhizobacteria and their mechanisms of action.

2.1 Physiochemical characterization of soil sample

Parameters	Measurements
pH	7.8
Salinity	0.18
Ec (mmhos/cm)	0.36
(TOC %)	0.63
Nitrogen (Kg/ha)	123
Phosphorus (ppm)	83
Cu^{2+} (ppm)	171

Zn^{2+} (ppm)	0.46
Mn^{2+} (ppm)	1.04
Fe^{2+} (ppm)	1.23
Texture	Loamy Soil
Colour	Brown
Temperature	34°C

2.2 Isolation of Rhizobacteria on basis of Biocontrol Attributes

2.2.1 Production of Siderophores by screened isolates

Among 14 screened isolates, 5 showed siderophore production on CAS agar, with isolates D5 and A1 exhibiting the highest production (75% and 65% units, respectively). These isolates were selected for further biocontrol trait analysis due to their superior siderophore production, a key factor in iron acquisition and pathogen resistance. Quantitative analysis confirmed their potential for managing biotic stress.

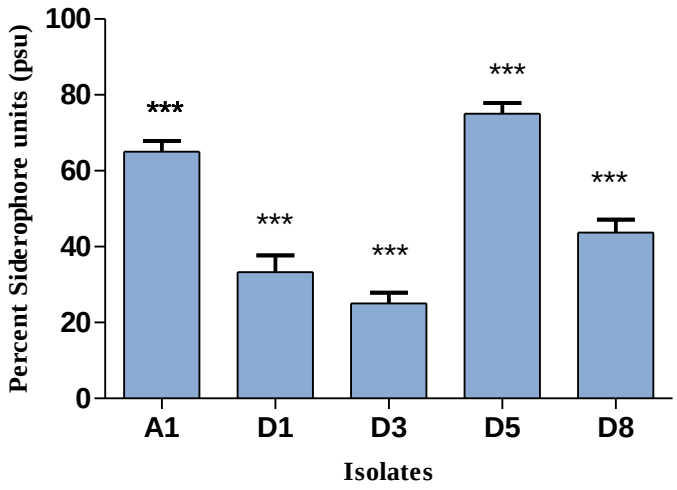


Figure 2.1: Siderophore production by selected bacterial isolates, with significance levels indicated (ns = $p > 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$), based on mean values from three replicates ± standard error

2.2.2 Production and characterization of EPS

Among 14 isolates, 5 produced notable exopolysaccharides (EPS) on sucrose-supplemented media, with D5 yielding 0.7% and A1 yielding 0.5% (w/v) EPS after 96 hours. Both isolates showed slightly acidic pH (6), moderate flocculating activity (D5: 12%, A1: 14%), and similar viscosity (~0.8 mPas). While D5 had higher carbohydrate content (0.45 g%) and EPS yield, A1 exhibited higher protein content (0.12 g%). These traits highlight their potential in abiotic stress tolerance and root colonization.

Table 2.1: Characterization of EPS recovered from isolate A1 and D5

Sample	**A1**	**D5**
Viscosity (mPas)	0.82; 26°C	0.87; 26°C
Total Carbohydrate (g %)	0.31	0.45
Total protein (g %)	0.09	0.09
Flocculating Activity	14	12
EPS yield (g %)	0.5	0.7

2.2.3 Biocontrol Attributes of selected isolates A1 and D5

The antagonistic activity of isolates D5 and A1 against *Cercospora canescens* was evident, with D5 showing the highest fungal growth inhibition (71.4%) and a significant reduction in fungal biomass (0.6 g) compared to A1 (69.2% inhibition, 1.6 g biomass) and the control (11.6 g biomass). Both isolates demonstrated efficacy in dual and liquid culture methods, with D5 emerging as the most effective, consistent with studies on Bacillus spp. against *Cercospora* pathogens.

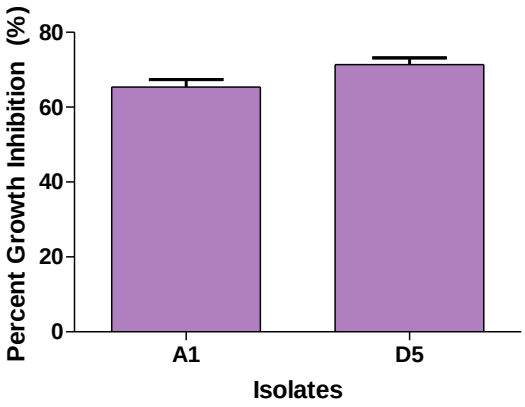


Figure 2.2: Antagonistic activty by isolates A1 and D5 against *Cercospora canescens*

Table 2.2: Hydrolytic enzyme activity by isolates A1 and D5

Isolate	Chitinase Activity (U/mL)	Cellulase Activity (U/mL)	Zone of Hydrolysis (mm)	Chitinase Zone (mm)	Cellulase Zone (mm)
D5	0.8 - 1	0.5 - 0.9	34.1	34.1	25.3
A1	0..8	0.5	24.3	24.3	23

2.2.4: Biofilm Formation by selected isolates

Biofilm formation by isolates A1 and D5 was assessed using test tube and microtiter plate method, with D5 demonstrating the highest biofilm formation and colonization potential, while A1 showed moderate biofilm-forming ability. This highlights the role of biofilm in root colonization and biocontrol efficacy against *C. canescens*, consistent with previous PGPR studies**.**

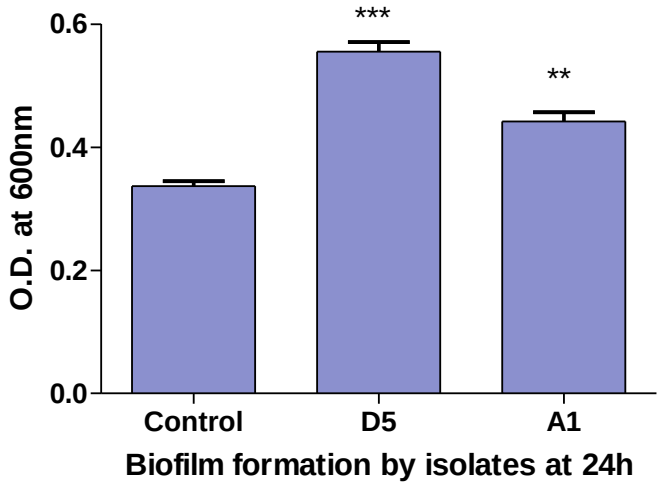


Figure 2.3: Biofilm formation by isolates A1 and D5 after 24h

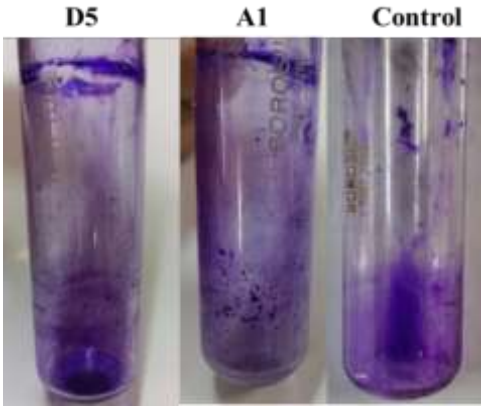


Figure 2.4: Biofilm formation on abiotic surface by isolates A1 and D5 compared to Control

Chapter 3: Characterization of Rhizobacteria for their plant growth promoting traits

Isolates A1 and D5 demonstrated multiple plant growth-promoting (PGP) traits, including indole acetic acid (IAA) production, ammonia production, and phosphate solubilization. Both isolates enhanced mung bean plant growth, with isolate D5 showing superior performance in root colonization and growth promotion, as confirmed through gnotobiotic assays. Antibiotic susceptibility testing revealed

that both isolates were resistant to multiple antibiotics, including β-lactams, macrolides, and aminoglycosides, with isolate D5 additionally resistant to norfloxacin. These resistance traits were utilized as genetic markers for bacterial tagging. Biochemical analysis, 16S rRNA gene sequencing, and phylogenetic studies identified isolate D5 as *Bacillus amyloliquefaciens* D5. The gene sequence was deposited in the GenBank database with the accession number OQ536061.

3.1 Biochemical Characterization, Carbohydrate Profiling and Antibiotic susceptibility tests of screened isolates

Attribute	Isolate D5	Isolate A1
Positive Biochemical Tests	Methyl Red, Voges-Proskauer, Citrate Utilization, IAA generation, Urea, Starch, Casein Hydrolysis, Ammonia production, Catalase, Dehydrogenase activity	Same as isolate D5
Carbohydrate Fermentation	Ferments all tested sugars	Ferments dextrose, sucrose, xylose, and maltose (restricted adaptability)
Antibiotic Resistance	Imipenem (10 µg), Norfloxacin (10 µg), Cefotaxime (30 µg), Erythromycin (15 µg), Kanamycin (30 µg), Ceftazidime (30 µg)	Amocillin (25 µg), Imipenem (10 µg), Cefotaxime (30 µg), Erythromycin (15 µg), Kanamycin (30 µg), Ceftazidime (30 µg)

3.2 Characterization of screened isolates for PGP traits

Phosphate solubilization -molybdenum blue method (Gaur 1990).

Ammonia production estimation – Nessler's reagent by Cappuccino and Sherman (1992).

IAA Production – Salkowsky's method (Sarwer and Kremer (1995).

Table 3.1: Plant growth promoting traits of selected isolates A1 and D5

Attribute	**Isolate D5**	**Isolate A1**
Phosphate Solubilization (Qualitative)	Zone size: 40 mm	Zone size: 30 mm
Phosphate Solubilization (Quantitative)	69.3 µg/mL of TCP solubilized	50.4 µg/mL of TCP solubilized
IAA Production (48h)	8.25 µg/mL	7.2 µg/mL
IAA Production (72h)	6.1 µg/mL	4.36 µg/mL
IAA Production (96h)	1.75 µg/mL	1.34 µg/mL
Ammonia Production	3.8 µg/mL	3.3 µg/mL

3.3 Gnotobiotic assay of selected isolates to study root colonization

The gnotobiotic assay showed significant growth enhancement in plants treated with isolate D5, evidenced by increased root and shoot length, followed by isolate A1, while control plants exhibited the least growth. The superior performance of D5 is linked to its nutrient uptake potential, IAA production, and beneficial root interactions.

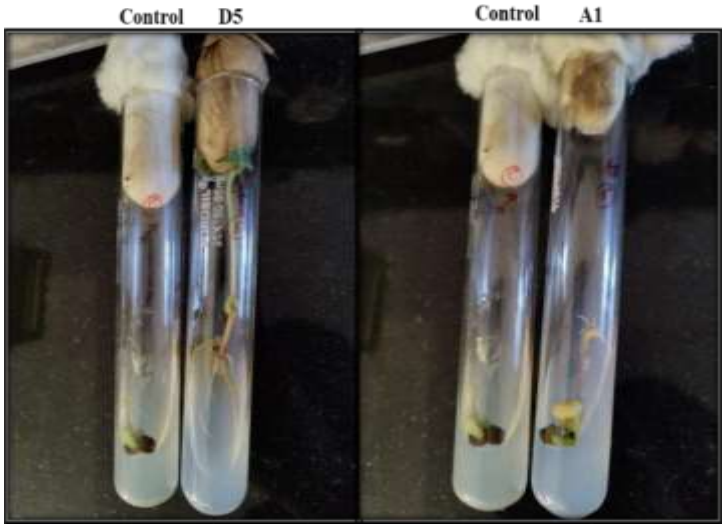


Figure 3.1: Gnotobiotic assay on MS media by screened isolates A1 and D5 comapred to control

3.4 Phylogenetic analysis of selected isolate

Isolate D5 was selected for identification due to its superior root colonization ability, antifungal activity, and plant growth-promoting traits.

99
80
40
37
75
23
46
62
NR 116022.1 Bacillus amyloliquefaciens strain BCRC 11601 16S ribosomal RNA partial sequence
D5
NR 112685.1 Bacillus amyloliquefaciens strain NBRC 15535 16S ribosomal RNA partial sequence
NR 041455.1 Bacillus amyloliquefaciens strain NBRC 15535 16S ribosomal RNA partial sequence
NR 151897.1 Bacillus nakamurai strain NRRL B-41091 16S ribosomal RNA partial sequence
NR 024696.1 Bacillus vallismortis strain DSM 11031 16S ribosomal RNA partial sequence
NR 113994.1 Bacillus vallismortis strain NBRC 101236 16S ribosomal RNA partial sequence
NR 102783.2 Bacillus subtilis subsp. subtilis strain 168 16S ribosomal RNA complete sequence
NR 180796.1 Bacillus stercoris strain JCM 30051 16S ribosomal RNA partial sequence
NR 180419.1 Bacillus cabrialesii strain TE3 16S ribosomal RNA complete sequence
NR 075005.2 Bacillus velezensis strain FZB42 16S ribosomal RNA complete sequence

Figure 3.2: Phylogenetic analysis of 16S rRNA sequences using the maximum likelihood method in MEGA-X with ClustalW alignment. Bootstrap values (1000 replications)

Chapter 4: *In planta* studies to check the efficacy of root colonization ability of screened isolates

In pot studies, *Bacillus amyloliquefaciens* D5 significantly enhanced seed germination (92.3%), vigour index (2193.2), and reduced disease incidence (26.6%) under *Cercospora canescens* stress compared to untreated controls, which showed 73% germination, a vigour index of 1496.5, and 83% disease incidence. Growth parameters, including root length, shoot length, biomass, and chlorophyll content, were notably improved, with isolate D5 outperforming A1 in all measures.

Field trials further demonstrated the efficacy of D5, showing an 80.17% increase in root length, 32.76% in shoot length, 53.34% in chlorophyll content, and 54.55% in pod number compared to untreated controls. This consistent superior performance underscored the potential of D5 as a bioinoculant for sustainable agriculture.

To extend the shelf life of rhizobacteria, bioformulations of D5 were developed using talc, cocopeat, chitosan, and charcoal as carriers. Talc-

and chitosan-based formulations effectively sustained the rhizobacterial population for up to six months. The talc-based formulation achieved the highest efficacy, with a germination rate of 94%, a vigour index of 2334, an 84% increase in fresh weight, a 66% increase in dry weight, and an 81% improvement in shoot length under stress conditions, while reducing disease incidence to 27.3%. The chitosan-based bioformulation also performed strongly, with a 93.3% germination rate, a vigour index of 2350.2, and a 30% increase in chlorophyll content, indicating enhanced photosynthesis. Charcoal and cocopeat formulations showed moderate benefits, with germination rates of 91.7% and disease incidences of 33.7% and 28.7%, respectively. These results highlight the effectiveness of talc and chitosan as carrier materials for *Bacillus amyloliquefaciens* D5 in enhancing plant growth and disease resistance in *Vigna radiata*.

4.1 Greenhouse study

In the pot experiments, isolate A1 showed moderate improvements in plant growth compared to the control. The germination percentage and vigour index for A1 were lower than D5, with a germination rate of 73% and vigour index of 1496.5. However, A1 still enhanced root length (96.4% increase), shoot length (56.3% increase), fresh weight (16.56% increase), dry weight (48.99% increase), and chlorophyll content (61.89% increase) compared to the control. Although its biocontrol efficacy was not as high as D5, A1 still contributed to reducing disease incidence by 13.4% compared to the control.

Table 4.1: Vegetative parameters measured under different treatments after 30 DAS of greenhouse study

Treatments	**Root Length (cm)**	**Shoot Length (cm)**	**Chlorophyll (ug/ml)**	**Dry weight (g)**	**Fresh weight (g)**	**Leaf spot Severity Index**
Control (T1)	2.8±0.15b	13.02±0.07b	9.82±0.33b	0.457±0.01b	1.63±0.08b	0
C. canescens (T2)	0.3±0.07c	10.7±0.64c	5.6±0.31c	0.189±0.05c	0.431±0.03c	9±0.33
D5 (T3)	6.2±0.341a	24.6±0.47a	17.96±0.42a	1.055±0.08a	2.045±0.06a	1±1.2
D5+*C. canescens* (T4)	4.83±0.421a	17.7±0.18a	14.27±0.17a	0.548±0.01a	0.981±0.03b	3±0.05
A1 (T5)	4±0.12a	22.1±0.54b	15.9±0.11a	0.681±0.11a	1.9±0.55a	1±1.3
A1+*C. canescens* (T6)	5.5±0.9a	12±0.03a	11.8±0.8b	0.237±0.02b	0.575±b	3±0.1
LSD 5%	0.943	1.304	1.166	0.2216	0.1662	1.38

4.2 Field Trials with isolates A1 and *Bacillus amyloliquefaciens* D5

In the field trials, treatments T2 and T3 significantly improved root and shoot length, with T2 showing the highest increases of 81.03% and 81.94%, respectively, compared to T1. Both treatments also resulted in higher chlorophyll content and pod numbers, with T2 leading to a 54.55% increase in yield. T3 showed a slightly lower improvement in all parameters but still outperformed T1.

Table 4.2: Effect of application of different treatments on vegetative growth of *Vigna radiata* L. after 70 DAS. The data presented in the table represents the means ± standard errors of these replicates

Parameters	**Control (T1)**	**A1 (T2)**	**D5 (T3)**
Root Length (cm)	11.6±0.58	17.6±0.47	21±1.6
Shoot Length (cm)	77.3±0.471	83.6±2.86	102±1.24
Chlorophyll (µg/mL)	24.3±0.72	35.1±1.1	37.2±0.57
No. of Pods	11±1.24	14±0.47	17±0.81

4.3 Bioformulation studies

The comparative study of different carrier materials as bioformulations was carried out exclusively with *Bacillus amyloliquefaciens* D5 due to its superior growth-promoting efficacy compared to isolate A1. D5 demonstrated significantly better performance across various growth parameters and productivity metrics in field trials. This highlights its potential as an effective bioinoculant for agricultural applications.

Table 4.3: Effect of different bioformulations on growth parameters of *Vigna radiata* plants. Results are expressed as means of three replicates± standard error

Treatments	Chlorophyll Content (ug/ml)	Fresh Weight (g)	Dry Weight (g)	Root Length (cm)	Shoot Length (cm)
Control (T1)	11.91 ± 0.50	0.209 ± 0.01	0.0727 ± 0.004	3.2 ± 0.1	15.06 ± 0.3
C. canescens (T2)	6.10 ± 0.70*	0.1523 ± 0.02*	0.0593 ± 0.005*	2.4 ± 0.2**	9.2 ± 0.5**
Talc (T3)	18.83 ± 0.70**	0.315 ± 0.01**	0.1973 ± 0.01*	4.6 ± 0.2**	22.1 ± 0.5**
Talc + *C. canescens* (T4)	14.55 ± 0.60**	0.24 ± 0.01b*	0.1107 ± 0.01**	5.16 ± 0.3**	18.1 ± 0.4*
Cocopeat (T5)	16.49 ± 0.70**	0.226 ± 0.01*	0.1237 ± 0.01*	4 ± 0.2**	18.1 ± 0.4**
Cocopeat + *C. canescens* (T6)	13.7 ± 0.60**	0.197 ± 0.01*	0.1093 ± 0.01**	3 ± 0.2**	14.3 ± 0.3**
Chitosan (T7)	20.34 ± 0.80**	0.2897 ± 0.01**	0.0533 ± 0.004*	4.93 ± 0.2*	16.23 ± 0.4*
Chitosan + *C. canescens* (T8)	14.44 ± 0.70**	0.277 ± 0.01**	0.0333 ± 0.004**	6.97 ± 0.3**	19.47 ± 0.5*
Charcoal (T9)	22.1 ± 0.90**	0.339 ± 0.01**	0.076 ± 0.005**	3.5 ± 0.2*	19.1 ± 0.5**
Charcoal +*C. canescens* (T10)	14.5 ± 0.70**	0.223 ± 0.01**	0.044 ± 0.004*	2.8 ± 0.2***	16.1 ± 0.44**

Chapter 5: Deciphering Siderophore Dynamics: Extraction, Purification, and their Impact on Defense Enzyme Activity and Gene Expression Modulation

Siderophores produced by *Bacillus amyloliquefaciens* D5 were optimized, purified using Amberlite XAD columns, and confirmed to be of catecholate nature via FTIR, HPLC, UV spectroscopy, LC-MS, and Arnow's test. A concentration of 50 µg/mL was quantified using the CAS assay. Application of purified siderophores to plants significantly improved growth parameters under stress, with an 81% increase in shoot and root length, 84% enhancement in fresh weight, and a reduction in *Cercospora* leaf spot disease incidence from 83% (control) to 27.3%. Defense enzyme assays revealed dose-dependent activation of key enzymes at different concentrations of siderophore from 5µg/mL to 15 µg/mL. Total phenolic content, peroxidase (PO), polyphenol oxidase (PPO), phenylalanine ammonia-lyase (PAL), and chitinase activities were markedly elevated, particularly in combined siderophore and pathogen treatments (SP15). Gel staining highlighted increased expression of PO and PPO isoforms, suggesting enhanced defense responses.

Protein analysis revealed distinct banding patterns, indicating induction of specific stress-responsive proteins (46–50 kDa, 32–46 kDa, 25 kDa, 22 kDa, and 17 kDa) in siderophore-treated plants. Gene expression studies showed substantial upregulation of catalase (CAT, up to 25.9-fold), epoxide hydrolase (EH, up to 77.3-fold), and PR-10, especially in combined siderophore and pathogen treatments, demonstrating a robust activation of antioxidant and defense pathways. These findings underscore the potential of siderophores from *Bacillus amyloliquefaciens* D5 in improving plant growth, reducing disease incidence, and enhancing plant defense mechanisms, offering a promising solution for sustainable agriculture.

5.1 Optimization, Purification and Characterization of siderophores

Siderophore production by *Bacillus amyloliquefaciens* D5 was optimized at pH 7, 30°C, and 50 µM iron concentration, with the highest yield observed under these conditions. Acidification of the supernatant to pH 4 facilitated effective binding to Amberlite XAD resin during column chromatography, resulting in a final siderophore concentration of 50 µg/mL. The CAS assay and calibration curve confirmed the significant production of siderophores**.** The purified siderophores were then characterized using different analytical techniques as shown below (Table 5.1).

Table 5.1: Characterization of purified siderophores by various techniques

Analysis	Key Findings	Supporting References
UV-Visible Spectroscopy	Absorption peaks at 254 nm, 264 nm, 303 nm, and 308 nm confirm catecholate-type siderophores.	Liu et al. (2023); Nithyapriya et al. (2021); Saiyyed et al. (2005)
Arnow's Test	Positive red coloration confirms the presence of catecholate moieties in the siderophore produced by *Bacillus amyloliquefaciens* D5.	Arnow (1937)
FTIR Analysis	Key absorption bands at 3396 cm-1 (O-H stretching), 2986 cm-1 (C-H stretching), 1733 cm-1 (C=O stretching), indicating the presence of catecholate groups, specifically 2,3-dihydroxybenzoic acid.	Chen et al. (2012); Nithyapriya et al. (2021)

HPLC Analysis	Purified siderophore confirmed with a peak at 2.5 minutes, identical to standard 2,3-dihydroxybenzoic acid, indicating no contaminants.	Rai et al. (2020)
LC-MS Analysis	Characteristic fragmentation peaks at m/z 217.35, 341.74, 415.37, 438.77, 514.90, 550.63, 571.92, 691.80, and 906.39 suggest the presence of bacillibactin, confirming the siderophore's structure.	Zhou et al. (2018); Sandy et al. (2009); Hertlein et al. (2014)

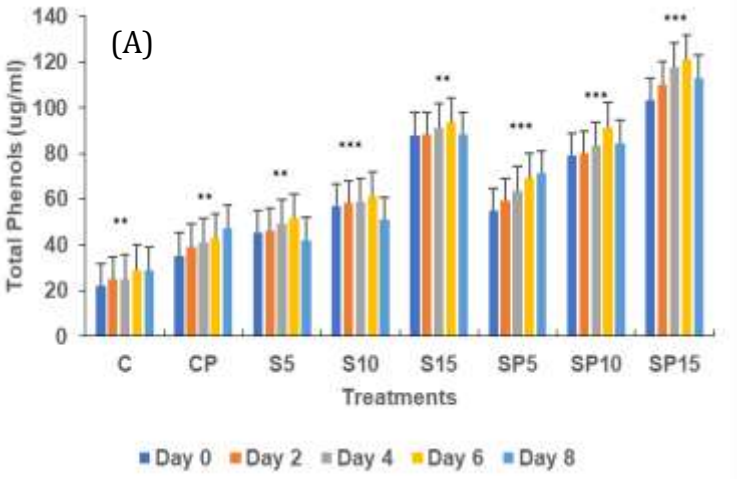
5.2 Plant growth promotion study using purified siderophores

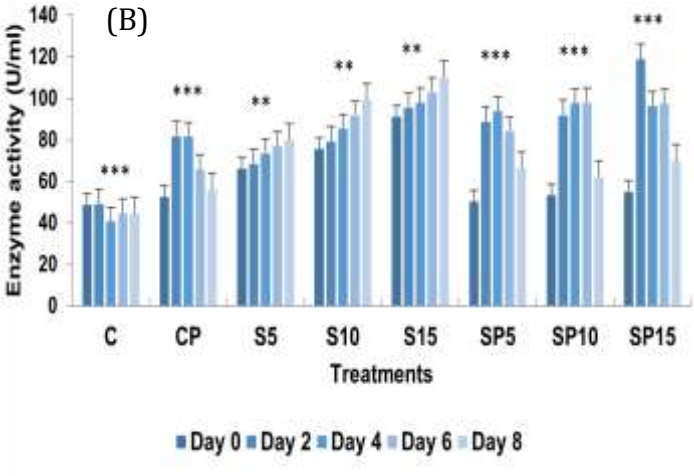
Vegetative Parameters	Control (T1)	Pathogen (T2)	Siderophore (T3)	Siderophore+ Pathogen (T4)	LSD (α= 0.05)
Root Length (cm)	3.8±0.571a	2.6±0.471b	6.5±0.408c	5.76±0.555c	1.2
Shoot Length (cm)	14.5±0.368a	12.5±0.368b	18.5±0.408c	22.4±0.418d	1.4
Chlorophyll (µg/mL)	18.965±0.265a	10.135±0.585b	15.985±1.485c	25.6±1.1d	3.1
Fresh Weight (g)	0.273±0.010a	0.237±0.006b	0.327±0.013c	0.385±0.007d	0.047
Dry Weight (g)	0.0534±0.0004a	0.0406±0.001b	0.054±0.002a	0.064±0.001c	0.045

5.3 Siderophore mediated defense enzyme assay

The study involved 8 treatments, with total phenols increasing in all except the untreated control. The highest phenol levels were found in treatments S15 and SP15.

Peroxidase, polyphenol oxidase (PPO), phenylalanine ammonia lyase (PAL), and chitinase activities were highest in treatments involving siderophores and *C. canescens,* indicating enhanced plant defense responses. The combination of siderophores and *C. canescens* generated a stronger immune response, leading to increased phenol production and enzyme activities.





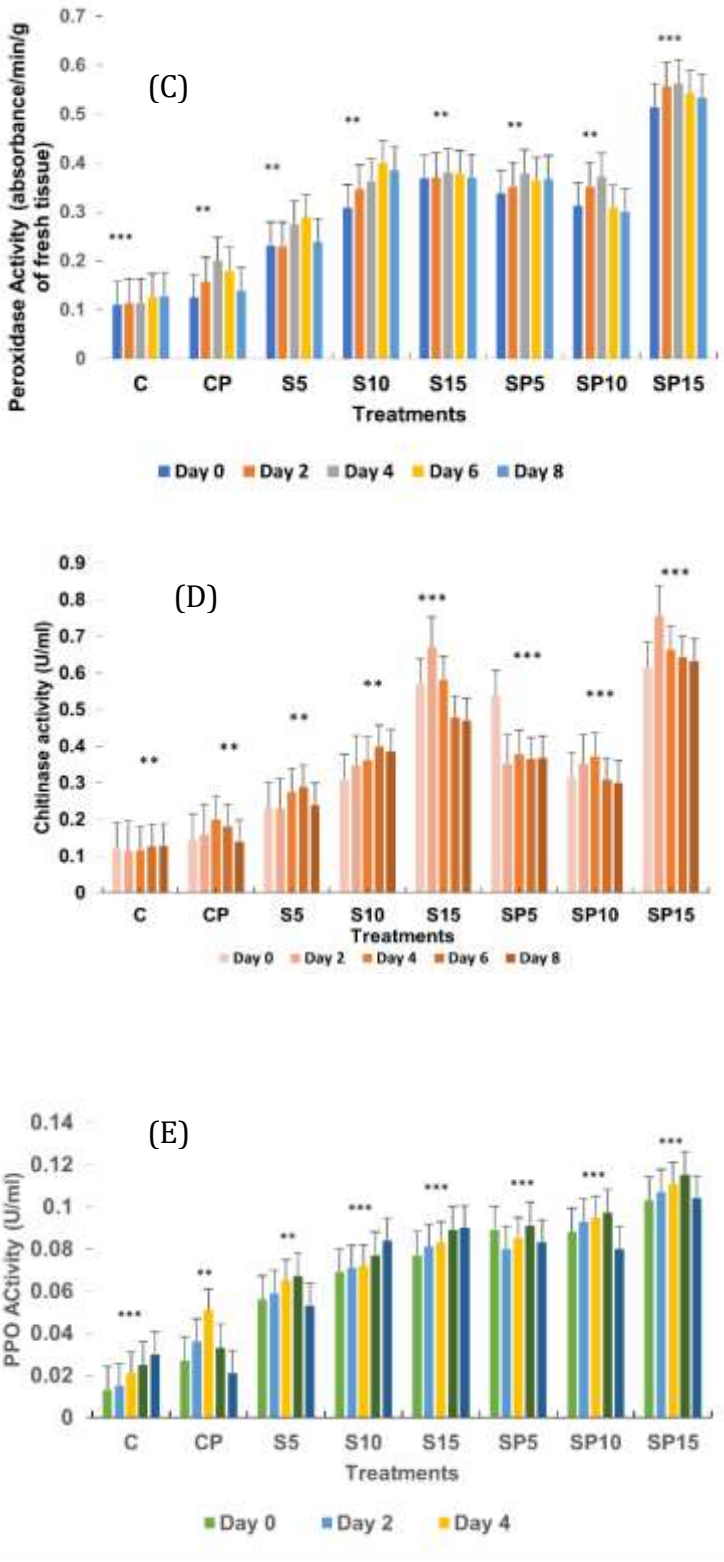


Figure 5.1: Defense enzyme assay using purified siderophores at different concentrations (A) Phenylalanine ammonia lyase (B) Total Phenols (C) Peroxidase (D) Chitinase (E) Polyphenol Oxidase

5.4 Native and SDS PAGE analysis of different treatments

The study revealed that protein banding patterns in plants treated with siderophores and *C. canescens* showed similar bands, indicating a general stress response mechanism. Additional bands were observed in *C. canescens* treated plants. The response to siderophore treatment may involve cross-talk between defense mechanisms and nutrient signaling pathways. The combination of siderophores and *C. canescens* enhanced defense mechanisms, leading to overlapping banding patterns compared to individual treatments. The study found that PPO isoforms 1, 2, and 3 were present in all treatments, with some isoforms constitutively expressed in plants. C. canescens infection, siderophore treatment, or their combination induced changes in PPO expression levels or activity, but did not result in new isoforms. PO isoforms 1 and 2 were expressed in all treatments, while PO isoform 3 was only found in the control. The treatments likely activated specific isoforms relevant to the stress. response.

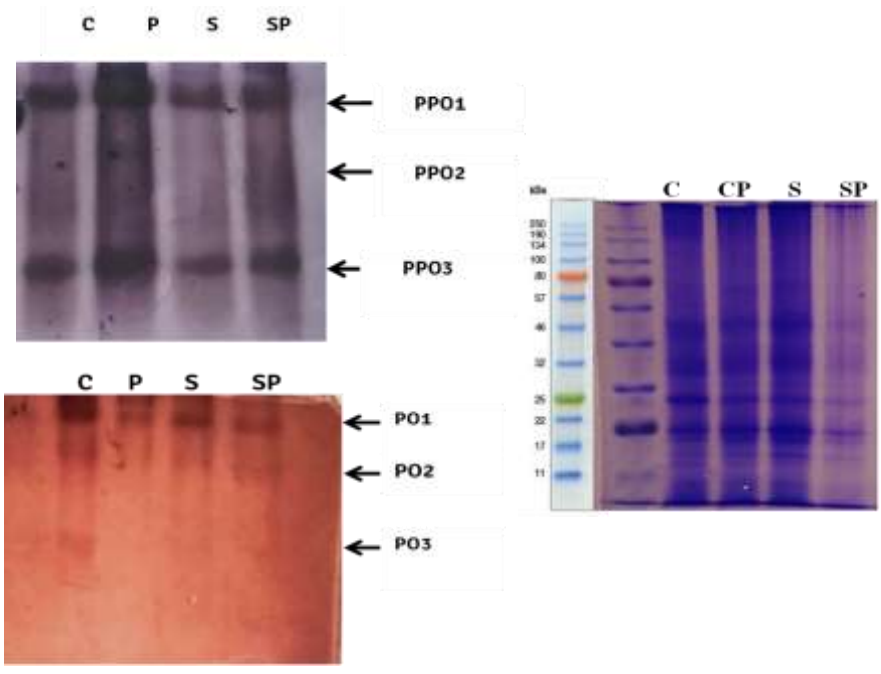


Figure 5.2: SDS and Native PAGE analysis of various treatments where C – control, P – pathogen, S – siderophore and SP – siderophore challenge inoculated with pathogen

5.5 Siderophore mediated gene expression studies

Catalase-mediated detoxification of ROS helps plants limit the spread of *C. canescens* by preventing oxidative damage and activating defense pathways. The CAT gene expression increased significantly in response to *C. canescens* and siderophore treatments, with the highest upregulation observed in the combined treatment. Epoxide hydrolase (EH) gene expression was also highly upregulated in response to both treatments, indicating a strong defense response. Calcium/calmodulin-dependent protein kinases (CCaMKs) and calmodulin-binding proteins regulate defense-related genes and enzymes, with higher expression observed in combined treatments. PR-10 proteins, associated with plant defense, were upregulated in siderophore and combined treatments, enhancing plant protection.

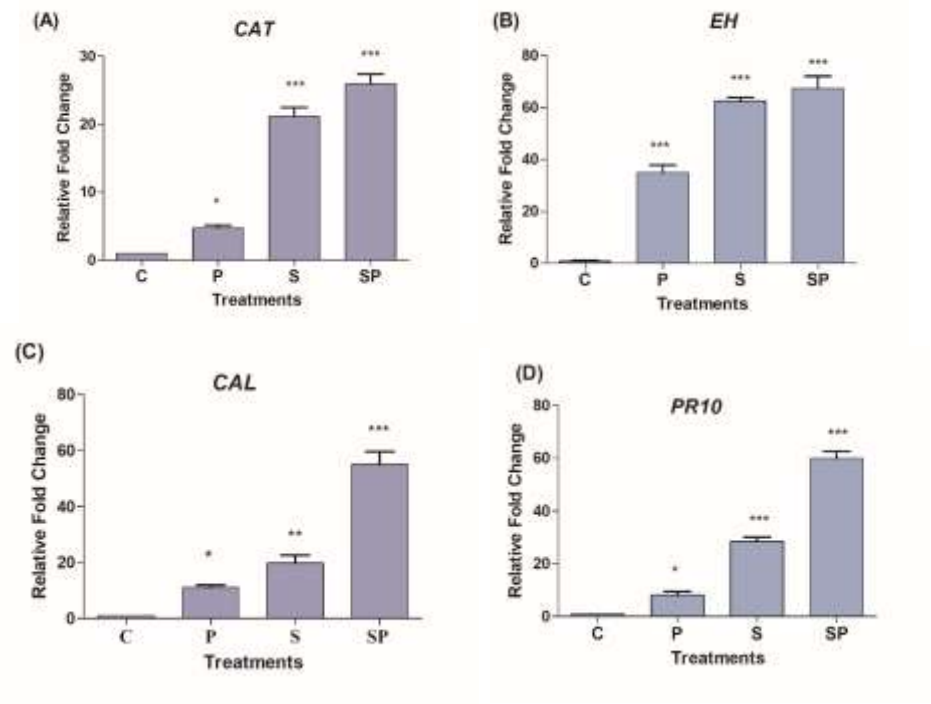


Figure 5.3: (A) Relative fold change of the Catalase (CAT) gene expression (B) Epoxide Hydrolase (EH) (C) Calmodulin (CAL) (D) PR10 gene expression under different treatments. Data represent mean values ± standard error, with ***, **, * indicating significant differences ($p < 0.001$, $p < 0.01$, $p < 0.05$, respectively) compared to the control condition

Chapter 6: Artificial Intelligence in Plant Health

Two AI models, Multilayer Perceptron (MLP) and Random Forest (RF), were employed for disease prediction. The MLP model demonstrated higher accuracy (91%) compared to RF (75%), emphasizing the importance of algorithm selection in effective plant health monitoring and disease prediction. A correlation matrix analysis identified strong positive relationships between shoot length, number of leaves, and leaf length, highlighting their significance as growth indicators. Sunlight positively influenced these parameters, underscoring its role in promoting plant development, while daytime temperature also supported optimal leaf growth. However, negative correlations between sunlight and humidity indicated potential challenges in controlled environments, necessitating the careful management of environmental factors to enhance plant growth and productivity.

6.1 Comparison of two AI models in predicting disease severity

The comparison between Random Forest (RF) and Multilayer Perceptron (MLP) models shows that RF outperforms MLP, with an accuracy of 91.30% compared to MLP's 75%. RF demonstrates high precision across all classes, while MLP has high precision only for the low class and zero precision for medium and high classes. RF is effective for multi-class classification, handling imbalanced datasets, and minimizing misclassifications. Further optimization of RF parameters and integration with feature selection techniques could enhance medium-class identification.

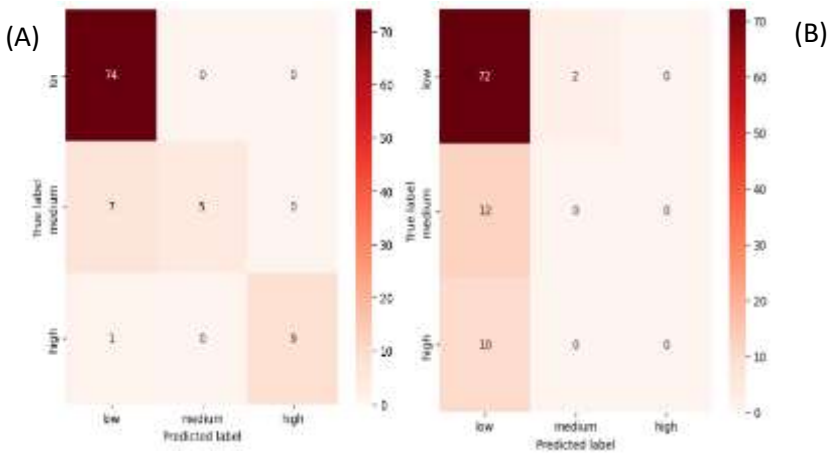


Figure 6.1: Comparison of two AI models (A) Random Forest (B) Multilayer Perceptron in prediction of disease severity

6.2 Correlation Matrix in feature selection

The correlation matrix highlights key relationships between plant growth variables. Strong positive correlations exist between Shoot Length, Number of Leaves, and Leaf Length, indicating overall plant health. Sunlight positively influences these growth factors, while Temperature (day) supports leaf development. Treatment and Dose show a weak positive correlation, suggesting higher doses may impact growth. Negative correlations are found between Sunlight and Humidity, and Sunlight and Temperature (night), indicating environmental influences on plant growth. Managing sunlight, temperature, and treatment doses is crucial for optimizing plant growth and productivity.

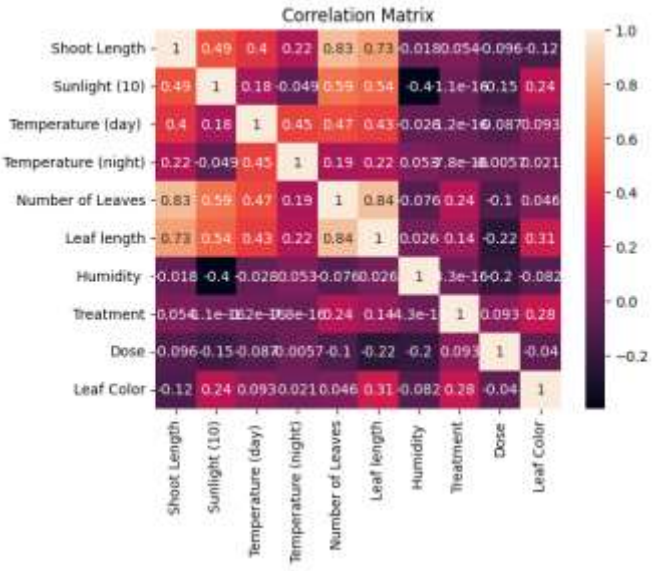


Figure 6.2: Correlation matrix highlighting feature selection importance in disease severity prediction

Conclusions

The study focused on isolating rhizobacteria from mung bean plants, specifically from the Vagadkhol area near Ankleshwar, Gujarat. Out of 28 initial isolates, 14 motile isolates were screened for their ability to produce siderophores and exopolysaccharides (EPS). Two isolates, A1 and D5, demonstrated significant biocontrol potential against *Cercospora canescens.* Isolate D5, identified as *Bacillus amyloliquefaciens*, showed superior performance in promoting plant growth and reducing disease incidence compared to isolate A1. Field trials confirmed the efficacy of *Bacillus amyloliquefaciens* D5 in enhancing growth parameters and reducing disease incidence. Gene expression analysis revealed significant upregulation of defense-related genes, such as catalase, epoxide hydrolase, and PR-10, in response to siderophore and pathogen treatments, indicating a robust activation of plant defense mechanisms. Bioformulation studies highlighted the effectiveness of talc and chitosan-based formulations in sustaining the rhizobacterial population and enhancing plant growth and disease resistance. Purified siderophores from *Bacillus amyloliquefaciens* D5 significantly improved plant growth parameters and reduced disease incidence, showcasing their natural

biocontrol potential. The study also compared AI models for disease prediction, with the Multilayer Perceptron model achieving higher accuracy than the Random Forest model. The correlation matrix revealed strong positive relationships between shoot length, number of leaves, and leaf length, indicating their importance as key indicators of plant growth. Sunlight and daytime temperature positively influenced these growth parameters, while negative correlations between sunlight and humidity highlighted potential challenges in controlled environments. Overall, the study underscores the potential of *Bacillus amyloliquefaciens* D5 as an effective bioinoculant for enhancing plant growth and disease resistance, with significant implications for sustainable agriculture. The findings emphasize the importance of managing environmental factors and optimizing treatment doses to maximize plant growth and productivity. The research also highlights the critical role of algorithm selection in enhancing plant health monitoring and disease prediction, with the Multilayer Perceptron model proving to be more effective than the Random Forest model in this context.

List of Publications

- Kamath A., Sharma A., Shukla A, Parmar P, Patel D., (2024) Fortifying plant fortresses: siderophores in defense against Cercospora leaf spot disease, Frontiers in microbiology (Accepted)

- Kamath A., Patel S., Patel D., (2024) Microbial allies: siderophores from *Bacillus amyloliquefaciens* D5 enhances *Vigna radiata L.* growth, Indian Phytopathology, https://doi.org/10.1007/s42360-024-00806-1
- Kamath A., Patel D., (2024) Harnessing the Nature's Arsenal: *Bacillus amyloliquefaciens* D5 as a Biocontrol agent against Cercospora Leaf Spot Disease, Physiological and Molecular Plant Pathology (Communicated)
- Kamath, A., Shukla, A., & Patel, D. (2023), Quorum Sensing and Quorum Quenching: Two sides of the same coin. *Physiological and Molecular Plant Pathology*, *123*, 101927. https://doi.org/10.1016/j.pmpp.2022.101927
- Kamath, A., Shukla, A., Saiyed, T., Bhatt, S., Rathod, H., Makwana, V., Soni, D., Banerjee, S., & Patel, D. (2023), Bioinoculants: the agrarian avengers. *Symbiosis*, *91*(1–3), 151–166. https://doi.org/10.1007/s13199-023-00953-5
- Kamath, A., Patel, D., & Shukla, A. (2022), Traversing through the intricate interplay of plant microbiome front runners. *European Journal of Plant Pathology*, *165*(3), 407–419. https://doi.org/10.1007/s10658-022-02618-3

- Patel, D., Mamtora, D., Kamath, A., & Shukla, A. (2022). Rogue one: A plastic story. *Marine Pollution Bulletin*, *177*, 113509. https://doi.org/10.1016/j.marpolbul.2022.113509
- Shukla, A., Jani, N., Polra, M., Kamath, A., & Patel, D. (2021). CRISPR: The Multidrug Resistance Endgame? *Molecular Biotechnology*, *63*(8), 676–685. https://doi.org/10.1007/s12033-021-00340-9
- Patel S., Kamath A., Patel D., (2022) Quirky Perks of Diazotrophs, Vidya Journal, Vol 17. ISSN:2321-1520, Gujarat university

Conferences and Workshops

- Poster Presentation on "Siderophores as promising tools for empowering *Vigna radiata L.* against Cercospora Leaf Spot Disease" at Annual Research Conclave (2024) Institute of Advanced Research, Gandhinagar, Gujarat
- Oral Presentation on "Rhizobacterial Determinants mediated elicitation of plant defense mechanisms" in National Conference on Emerging Paradigms in Agricultural Microbiology, Atmiya University, Rajkot, Gujarat (2023) (2nd prize)
- Poster Presentation on Evaluating Siderophores as potential ISR determinant for empowering *Vigna radiata L* against Cercospora Leaf Spot Disease in National Conference on Present Dat Biology at St. Xavier's College, Ahmedabad, Gujarat (2023) (1st prize)
- Poster Presentation on Invigoration of *Vigna radiata* L and Biocontrol against Cercospora Leaf Spot disease by Plant Growth promoting bacterium *Bacillus amyloliquefaciens* D5 in 63rd Annual International Conference of Association of Microbiologists of India (AMI) at MDU, Rohtak, Haryana (2023)
- Workshop (5 days) on Outcome based teaching and learning practices and research (2023) by IIC at Institute of Advanced Research.
- Workshop on (5 days) Intellectual Property Rights and Innovations, by IIC at Institute of Advanced Research.
- Poster Presentation on Evaluating Siderophores as promising tools for empowering *Vigna radiata* L against Cercospora Leaf Spot Disease in 8th ASIAN PGPR society for sustainable agriculture National Conference on Beneficial Microbes for Integrated Plant Health Management at College of Horticulture, Bangalore, Karnataka (2023)
- Oral Presentation on "Rhizobacterial Determinants Mediated Elicitation of Plant Defense Mechanism" at Annual Research Conclave (2023) Institute of Advanced Research, Gandhinagar, Gujarat (2nd prize)
- Oral Presentation on "Rhizobacterial Determinants mediated elicitation of plant defense mechanisms" in National Conference on Plant Microbe Interactions and Plant Genetic Engineering, Institute of Advanced Research, Gandhinagar, Gujarat (2022) (2nd prize)
- Offline Workshop on Basics of Bioinformatics, June (2022) at Nirma University, Ahmedabad

- Poster Presentation on “Rhizobacterial Mediated Elicitation of Plant Defense Mechanism” at Annual Research Conclave (2021), Institute of Advanced Research, Gandhinagar (2nd prize)
- National Level Virtual Symposium on Recent Trends in Modern Biology organized by Charusat university
- Online workshop on “Real Time PCR for Gene Expression and Pathogen Detection” organized by Sathyabama University, Chennai
- Online Workshop on Molecular Marker Technology for Crop Improvement organized by Sathyabama University, Chennai
- Online Workshop on “Techniques in Decoding DNA” organized by Sathyabama University, Chennai
- Online Workshop on Gel Based Proteomics organized by Amazing Biotech Pvt Ltd